Expression of Tyrocidine

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Content

1. Introduction Tyrocidine construct

Our goal was the expression of tyrocidine in *Bacillus subtilis*, a well-known model organism, in order to make tyrocidine accessible to further improvements by oligo mediated recombineering. With a size of 39.5 kb, the tyrocidine operon is too large for an amplification with standard PCR techniques. [1] Therefore we have made efforts to create a construct which contains a toxic gene cassette flanked by two sequences which allow insertion of the tyrocidine operon via homologous recombination. The toxic gene is placed under control of the Pxyl promotor, which is repressed by the xylose responsive repressor XylR in the absence of xylose (see BBa_K733002). If xylose is present, the repressor leaves the operator sequence. Thereupon Pxyl is active and the toxic gene is transcribed. The construct is flanked by two sequences homologous to the lacA sequence, which allow integration into the genome of *Bacillus subtilis*.

2. Methods &Materials

2.1. Preparation of parts

2.1.1. Amplification of parts by PCR

Parts were amplified by PCR reaction with Phusion® High-Fidelity DNA Polymerase according to the manufacturer's protocol, using template and primers as specified below.
The PCR products were run on a 1% agarose gel to verify their length. The products were purified using QIAgen PCR purification kit and the concentration was determined by nanodrop.

Materials:

- Quick-Load® Purple 2-Log DNA Ladder –New England Biolabs
- Gel Loading Dye, Purple (6x) –New England Biolabs
- Phusion® High-Fidelity DNA Polymerase –New England Biolabs
- Phusion® HF Buffer –New England Biolabs
- DMSO –New England Biolabs
- 10 mM dNTP mix –New England Biolabs
- QIAgen PCR purification kit
- Biometra Thermocycler

- Primers according to the tables in the protocol[1]
- DNA according to the tables in the protocol[2]

2.1.2. Digestion of pSBC13 plasmid backbone

The linearized plasmid pSB1CR was cut with EcoRI-HF and PstI. Samples were mixed according to the table below and incubated at 37°C, followed by heat inactivation at 80°C for 20 minutes. The reaction was verified by running the digested backbone on a 1% agarose gel.

Sample	Concentration (ug/mL)	Volume (uL)
NEB Buffer 2.1		8µL
EcoRI-HF		0.2
PstI		0.2
Linearized Plasmid DNA	25	8
H_2O		0.6
Total		10

Table: restriction digestion of pSBC13

Materials:

- EcoRI-HF –New England Biolabs
- PstI –New England Biolabs
- Linearized pSB1C3
- Buffer 2.1 –New England Biolabs

2.2. Gibson assembly

The individual parts were assembled by Gibson Assembly. The parts were mixed with Gibson Assembly Master Mix and incubated at 50°C according to the manufacturer's protocol.

Materials:

- Gibson Assembly ® Master Mix –New England Biolabs

[1] Sequence of the primers can be found here

[2] Sequence of ordered g-blocks can be found here

- Biometra Thermocycler

2.3. Transformation

The Gibson assembled construct was transformed into top10 *E.coli* according to NEB's instructions and 100 µL were plated on LB plates +CAM plates and incubated at 37°C overnight.

Materials:

- NEB 5-alpha Competent E.coli (High Efficiency) –New England Biolabs

2.4. Colony PCR

To determine whether the *E.coli* colonies were successfully transformed with the plasmid containing the assembled tyrocidine construct, a colony PCR was conducted. Individual transformants were suspended in 50 µL ddH_2O and lysed by keeping them at 99°C for 7 min. 0.5µL of the samples were used as template per 25 µL reaction mix and a PCR was conducted according NEB's instructions, using the primers VF2 and VR.

Sample	Volume 25 µL (uL)	Volume x20 (uL)
5x Phusion Buffer	5	100
10 mM dNTPs	0.5	10
boiled colony sample	0.5	
Primer VF 2 (25 mM)	0.5	10
Primer VR (25 mM)	0.5	10
Polymerase Phusion	0.3	7
H_2O	17.7	354
Total	25	500

Table: colony PCR mastermix

PCR set-up

initial denaturation	98 °C	30 s	
denaturation	98 °C	10 s	x 30
annealing	59 °C	15 s	
elongation	72 °C	2 min 30 s	

final extension	72 °C	5 min	

Table: colony PCR reaction conditions

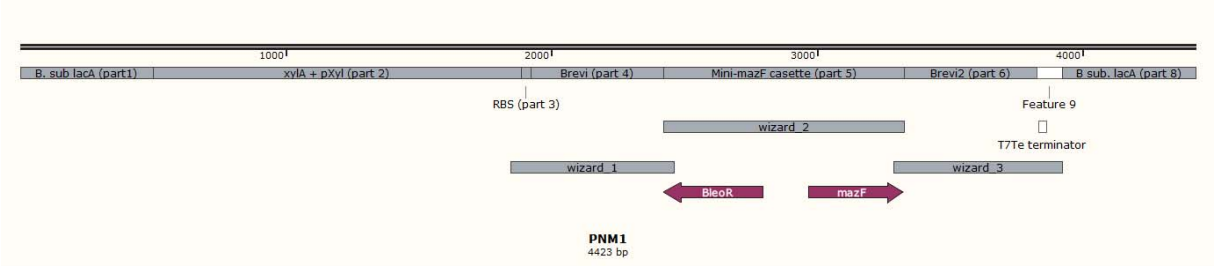
3. Initial Tyrocidine construct overview

3.1. Introduction

B. Sub recombination site	1
XylA(repressor) + Promoter(repressor) (BBa_K733002)	2
Ribosome Binding Site	3
Brevibacillus recombination (start codon)	4
Selection marker + mazF	5
Brevi recombination (stop codon)	6
Terminator	7
B. Sub recombination	8

Table: individual parts of the Tyrocidine construct

In the initial experimental design, we aimed at cloning all parts into one plasmid. Two constructs were designed: One containing an additional terminator (part 7) and one without additional terminator. The construct contains sequences homologous to *B.subtlis* lacA (part 1 +part 8), *Brevibacillus parabrevis* (part 5 +part 6), a selection marker and toxic gene cassette (part 5), as well as a xylose repressor and promotor (part 2), a ribosome binding site (part 3), and optional an terminator (part 7).



Picture 1: Tyrocidine with terminator assembled part 1 to 8 (taken from SnapGene)

3.2. Preparation of parts for Tyrocidine expression

The individual parts were amplified by PCR.

labelling	Part #	template	Primer	Annealing T.	size
A	pSB1C3 linearized	biobrick	priSB1C3_tyr-fwd	56°	~ 2kb
			priSB1C3_tyr-rev	54.7°	
B	Part 1	*B.subtilis* 168 gDNA	priTyr_part1-fwd	55.3°	~500 bp
			priTyr_part1-rev	54.8°	
C	Part 2	BBa_K733002	priTyr_part2-fwd		~1400 bp
			priTyr_part2-rev		
D	Part 8	*B.subtilis* 168 gDNA	priTyr_part8-fwd		~500 bp
			priTyr_part8-rev		
E	Part 6	Wizard3	priTyr_wizard_3-fwd		~500 bp
			priTyr_wizard_3-rev		
F	Part 8	*B.subtilis* 168 gDNA	priTyr_part8-2-fwd	65°	~500 bp
			priTyr_part8-rev	59°	

3.3. Tyrocidine construct Gibson assembly

The PCR amplified parts and the cut backbone pSB1C3 were assembled by Gibson assembly.

Construct 1 (with terminator)	**Construct 2 (without terminator)**
G-block: wizard_1	G-block: wizard_1
G-block: wizard_2	G-block: wizard_2
G-block: wizards_3	PCR: A
PCR: A	PCR: B
PCR: B	PCR: C
PCR: C	PCR: E
PCR: D	PCR: F

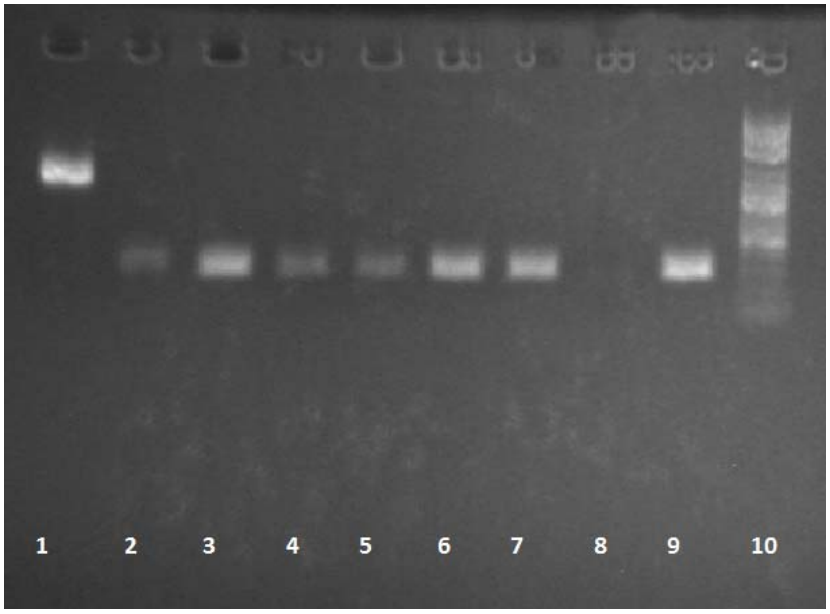
Table: parts used for the assembly of construct 1 and construct 2.

Tyrocidine construct I				
	concentration in ng/µL	length (bp)	ng to use	µL
a1	15.1	2070	44.4015	2.94
b	20.75	500	10.725	0.52
c	12	1400	30.03	2.50
d	24	500	10.725	0.45
gWizard1	10	614	13.1703	1.32
gWizard2	20	907	19.45515	0.97
gWizard3	10	635	13.62075	1.36
				10.06
Picomole vector				
0.0325				

Tyrocidine construct II				
	concentration in ng/µL	length (bp)	ng to use	µL
a1	15.1	2070	50.5494	3.35
b	20.75	500	12.21	0.59
c	12	1400	34.188	2.85
e	61	500	12.21	0.20
f	29	500	12.21	0.42
gWizzard1	10	614	14.99388	1.50
gWizzard2	20	907	22.14894	1.11
				10.01
Picomole Conc. Of vector				
0.037				

3.4. Tyrocidine construct Transformation and colony PCR

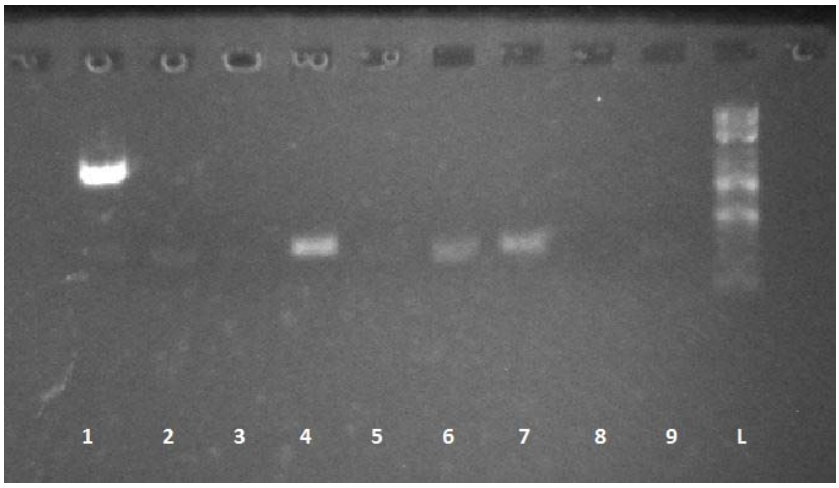
Tyrocidine construct I:



Gel: 08.08. tyrocidine construct I

lane 1	positive control
lane 2-9	colony samples of colonies 1-8 (from left to right)
lane 10	ladder

Tyrocidine construct II



gel: 08.08. tyrocidine construct II

lane 1	positive control
lane 2-9	colony samples of colonies 1-8 (from left to right)
lane 10	ladder

3.5. PCR troubleshooting

We further tried to amplify the cassette by amplifying the tyrocidine construct by running a PCR directly on the Gibson assembled product. The primers VR and VF2 were used. The bands were smaller than they should have been in the case of a correct assembly.

Primer 1	Primer 2	Size (bp)	Annealing T	Purpose
priTyr_part1-fwd	priTyr_part1-rev	500	58	Verify presence of part 1
priTyr_part1-fwd	priTyr_part2_rev	1900	58	Verify assembly of part 1 and 2
priTyr_part2_fwd	priTyr_part2_rev	1400	58	Verify presence of part 2
priSEQ-Tyr-3	priMini-Maz-BioB-rev	1650	58	Verify assembly of part 2-5
priSEQ-Tyr-4	priSEQ-Tyr-5	1200	60	Verify assembly of part 4-6
priSEQ-Tyr-4	priTyr_part8-rev	2000	58	Verify assembly of part 8 with

				construct
priMini-Maz-BioB-rev	priMini-Maz-BioB-rev	900	60	Verify presence of wizard 2
priTyr_part8-fwd	priTyr_part8-rev	500	58	Verify presence of part 8
VF2	priTyr_part1-rev			
VF2	priTyr_part2_rev			
VR	priTyr_part8-fwd			

3.6. Results and Conclusion:

The digest of the miniprep and the colony PCR showed that the assembly did not work as expected. The bands observed by colony PCR were less than 2 kb in size, while the construct should be around 4.8 kb in size. In addition a PCR was run with the primers VF2 and VR2 but the bands were smaller than expected.

PCR reactions with different primers were run in order to identify the problem. Part 3, 4, 5, 6, 7 and 8 seemed to be assembled while part 2 could not be amplified. Some more PCRs today that showed that we could amplify from pSB1C3 into the Biobricks on either site, suggests that part 2 did not get assembled into the construct.

4. Tyrocidine expression by splitting up construct

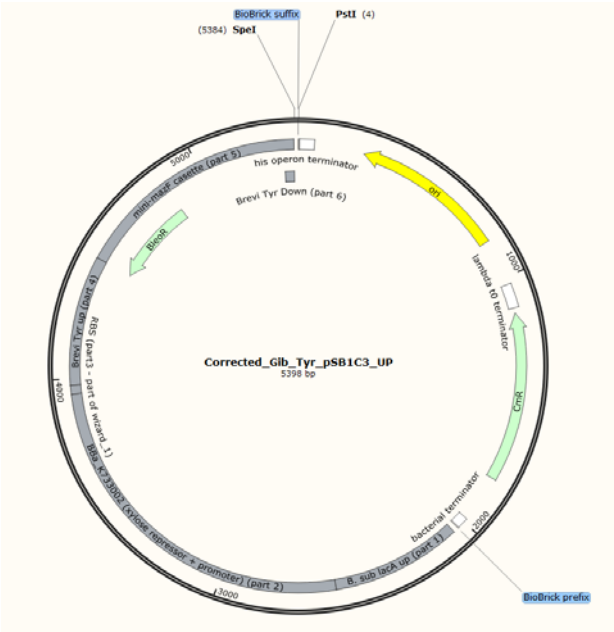
4.1. Intro

As the first attempt to express tyrocidine did not work and the efficiency of Gibson assembly drops as the assembly size increases, an attempt was made to express the construct by dividing the parts up into two plasmids.

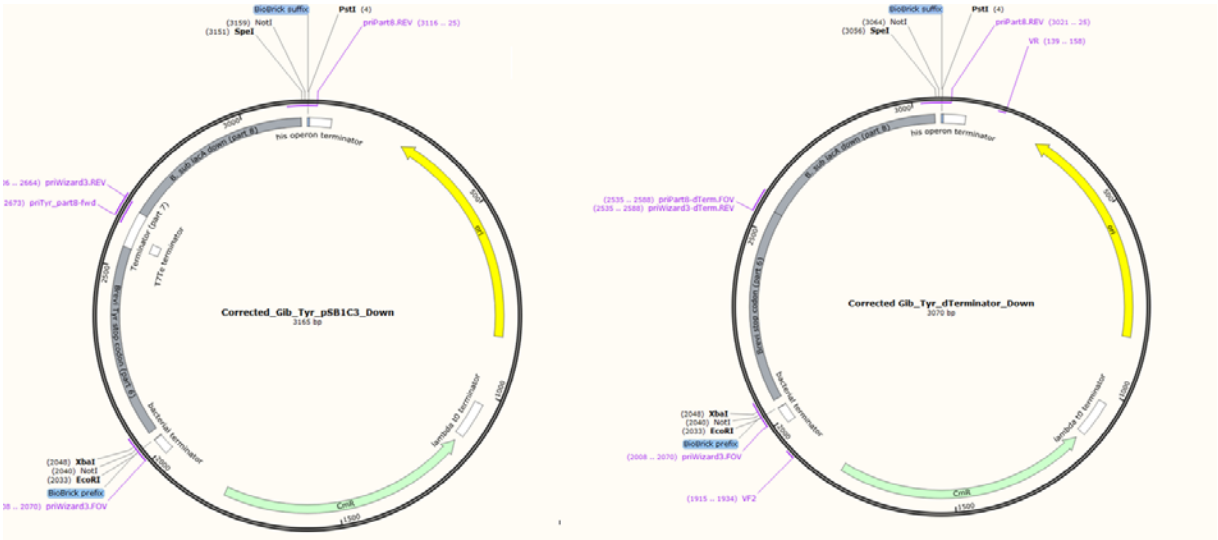
The “UP” construct was designed to contain the parts one to five in the pSB1C3 backbone, while the other parts were expressed either as “DOWN with Terminator” containing parts six, seven and eight in the pSB1C3 backbone or as “DOWN w/o Terminator” which was lacking part seven.

The constructs were designed in a way so that after successful assembly, they could be cloned to create the entire construct by ligating the “UP” construct digested with the restriction enzymes SpeI and PstI and the “DOWN with Terminator” or “DOWN w/o Terminator” digested with the restriction enzymes XbaI and PstI.

B. Sub recombination	1
XylA(repressor) + Promoter(repressor) (BBa_K733002)	2
RBS	3
Brevibacillus recombination (start codon)	4
Selection marker + mazF wizard_2	5
Brevi recombination (stop codon)	6
Terminator	7
B. Sub recombination	8



"UP" construct, taken from SnapGene



Down construct: left: Down construct with Terminator containing parts six, seven and eight. Right: Down construct without Terminator containing parts six and eight.

4.2. Preparation of parts for Tyrocidine expression

The individual parts were amplified by PCR reaction.

labelling	Part #	template	Primer	Annealing T.	size
A	Part 1	B.*subtilis* 168 gDNA	priPart1.FOR	57°	567 bp

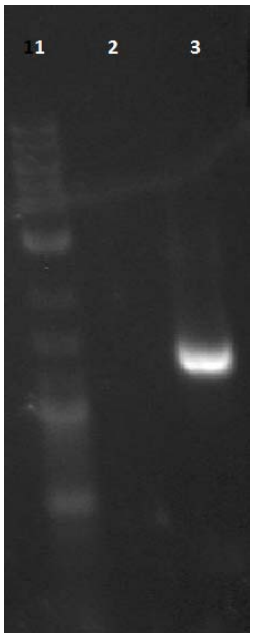
			priTyr_part1-rev	53°°	
B	Part 2	BBaK733002	priTyr_part2-fwd	55°	~1400 bp
			priTyr_part2_rev	56°	
	Part 3 +4	wizzard1	no PCR, added directly		
C	Part 5	Wizard2	priPart_Wizard2.FOR	59°	948 bp
			priPart_Wizard2.REV	54°	
D	Part 6 +7	Wizard3	priWizard3.FOV	65°	657 bp
			priWizard3.REV	60°	
E	Part 6	Wizard3	priWizard3.FOV	65°	567 bp
			priWizard3-dTerm.REV	59°	
F	Part 8 (with Term.)	*B.subtilis* 168 gDNA	priPart8.rev	56°	573 bp
			priTyr_part8-fwd	54°	
G	Part 8 (w/o Term.)	*B.subtilis* 168 gDNA	priPart8.rev	56°	561 bp
			priPart8-dTerm.FOV	59°	

4.3. Tyrocidine construct Gibson assembly

The PCR amplified parts and the cut backbone pSB1C3 were assembled by Gibson assembly.

UP-construct	**Down construct with T**	**Down construct w/o T**
Cut plasmid	Cut plasmid	Cut plasmid
A =part 1	D	E
B =part 2	F	G
Wizard 1		
C =part 5 (w2)		

4.5. Results and Discussion



lane 1	marker
lane 2	UP-construct
lane 3	DOWN-construct

The Down construct could be successfully assembled. The length was verified by amplifying the sample with the primers VF2 and VR and running it on a 1 % agarose gel.

5. Tyrocidine Expression with XylR in separate plasmid

5.1. Intro

Since it seemed like the xylose repressor was the piece that was not assembling, another construct was designed, which does not include xylR. XylR could then be expressed in a separate plasmid.

This is the individual DNA parts:

B. Sub recombination	1
Promoter constitutive	2

Brevibacillus recombination (start codon)	3
Selection marker + mazF wizard_2	4
Brevi recombination (stop codon)	5
Antibiotic reistance (zeocine)	6
B. Sub recombination	7

Use any of the g-blocks lambda beta, gp35 for promoter

5.2. Preparation of parts for Tyrocidine expression

The individual parts were amplified by PCR.

labelling	**Part #**	**template**	**Primer**	**Annealing T.**	**size**
A	Part 1	*B.subtilis* 168 gDNA	Fragment1.FOR	60°	537 bp
			Fragment1.REV	58°	
B	Part 2	gp35 in BB plasmid	Fragment2.FOR	60°	178 bp
			Fragment2.REV	60°	
C	Part 4	Wizard2	Fragment4.FOR	60°	508 bp
			Fragment4.REV	60°	
D	Part 5	Wizard3	Fragment5.FOR	68°	450 bp
			Fragment5.REV	60°	
E	Part 6 (w/o Term)	Wizard2	Fragment6.FOR	60°	567 bp
			Fragment6.REV	60°	
F	Part 7	*B.subtilis* 168 gDNA	Fragment7.FOR	59°	573 bp
			Fragment 7.REV	58°	
3	Part 3	Wizard1	Fragment3.FOR	59°	596 bp
			Fragment3.REV	60°	
G	Xylose Repressor	pDG268	pDG268neo-fwd-gibson	55°	~6000 bp
			pDG268neo-rev-gibson	54°	
H	Plasmid for XylR	BBaK733002	xylR_fwd	57°	~1200 bp
			xylR_rev	55°	

Table: template DNA, primers, fragment size and annealing temperatures (taken from SnapGene) of all fragments.

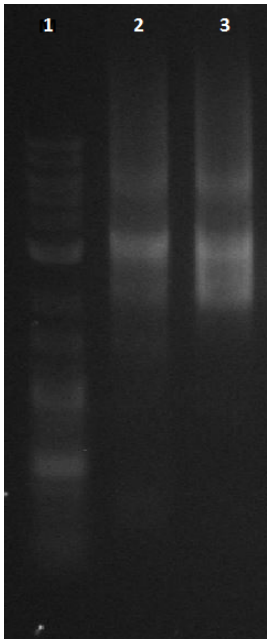
The reaction and correct size of all fragments was verified by running the samples on a 1 % agarose gel.

5.3.1. Gibson assembly

The PCR amplified parts and the cut backbone pSB1C3 were assembled by Gibson assembly.

	[ng/µL]	length (bp)	µL	ng
	20	2070	3.42	68.31
Sample A	60	537	0.30	17.72
Sample B	40.5	178	0.15	5.87
Sample C	131	508	0.13	16.76
Sample D	141	450	0.11	14.85
Sample E	80	567	0.23	18.71
Sample F	74	573	0.26	18.91
Sample 3	102.7	596	0.19	19.67
H_2O			5.23	
Gibson MM			10	
total			20	

	[ng/µL]	length (bp)	µL	ng	pmole
G	36	6000	2.78	100	0.026
H	14	1200	2.86	40	0.051
H2O			4.37		
Gibson			10		
total			20		



Gel: Tyrocidine construct

lane 1	ladder
lane 2	Tyrocidine construct
lane 3	Tyrocidine construct

5.3.2. Gibson assembly with DMSO

	[ng/µL]	length (bp)	µL	ng	pmol
G	36	6000	2.78	100	0.026
H	14	1200	2.86	40	0.051
DMSO			0.20		
H2O			4.17		
Gibson			10		
total			20		

5.5. Results and Conclusion

With this new experimental design, all parts except for xylR could be successfully assembled and the size of the construct was verified by amplifying the construct by PCR and running the product on a gel. This suggests that is was not the size of the construct that prevented the assembly of the initial tyrocidine construct.
However, xylR could not be cloned into the plasmid by Gibson assembly.

6. Isolation of gDNA from Brevibacillus parabrevis, 31.07.15

Purpose:

Isolation of genomic DNA from *Brevibacilus parabrevis*

Materials:

- TNE buffer
- TNEX buffer
- lysozyme solution
- proteinase K
- 5 M NaCl
- 96 % EtOH
- TE
- Malt media
- Meat media

Procedure:

1. OD of samples was measured and the ones with the highest OD were chosen.
2. DNA isolation according to protocol “Isolation of chromosomal DNA from E.coli” from Grimberg J. et al 1989 with the following exceptions:
    - step 3: 2 hours incubation time (as recommended in protocol for isolation of *Bacillus lactis DNA*)

Data:

Grown in malt media: OD_{600} =1.87
Grown in meat media: OD_{600} =1.73

Results and Conclusion:

The samples grown in malt media had a higher OD.
The gDNA was stored at -20°C.

Brevibacilus parabrevis genomic DNA 1	3147.56 ng/µL	260/230: 1.99
Brevibacilus parabrevis genomic DNA 2	1797.92 ng/µL	260/230: 1.84
Brevibacilus parabrevis genomic DNA 3	2005.21 ng/µL	260/230: 2.01
Brevibacilus parabrevis genomic DNA 4	736.55 ng/µL	260/230: 1.30

7. Achievements and final conclusion

We have succeeded in assembling all parts needed for the expression of tyrocidine, except for xylR, which we have been unable to assemble with all our various experimental designs.

The repressor was amplified by PCR using the Biobrick BBa_K733002 as template. The BioBrick has been used by the iGEM team Hong Kong University of Science and Technology 2012 (described here), which indicates that the biobrick is functional.

If given more time, the tyrocidine construct could be amplified with primers containing restriction sites and cloned into a plasmid which already contains XylR by classic cloning techniques, like the BioBrick BBa_K733002 or BBa_K733018. This plasmid could then be further amplified in *E.coli* and transformed into a *B.subtilis* strain which already has the xylose repressor integrated into its genome.

8. References

1. Mootz, H.D., & Marahiel, M.A. (1997). The tyrocidine biosynthesis operon of Bacillus brevis: Complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. Journal of Bacteriology, 179(21):6843-50